Chapter 7

The Impact of Environmental Stresses in the Virulence Traits of *Listeria monocytogenes* Relevant to Food Safety

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Abstract

Listeria monocytogenes is a foodborne pathogen, which causes listeriosis disease among humans and other animal species. Infections in humans mainly occur in immunocompromised individuals and are caused by the consumption of ready-to-eat and raw food products contaminated with the pathogen. To ensure survival in nature, *L. monocytogenes* easily adapts to different environmental conditions, and that justifies the hurdles to prevent bacterial growth inside the food chain. Exposure to a single or multiple sublethal stresses, as those impaired by food processing, food matrices, and the gastrointestinal tract, can enhance tolerance of *L. monocytogenes* to stresses and increase its survival and pathogenesis. This chapter summarizes the current information on the adaptive response of *L. monocytogenes* to different stresses, namely (1) cold stress, (2) acid stress, (3) osmotic stress, (4) desiccation stress, and (5) high hydrostatic pressure, and the impact of these stresses on *L. monocytogenes* virulence. The objective is to provide the background information that is necessary for the development of scientifically sound control strategies to improve food safety and to reduce the uncertainty of microbial risk assessments, associated to limited knowledge on the behavior of cells capable to adapt and survive stresses.

Keywords: *Listeria monocytogenes*, stress response, virulence

1. Introduction

Listeria monocytogenes is a pathogenic bacterium capable of causing listeriosis disease in humans and other animals. *L. monocytogenes* has a ubiquitous distribution in the environment [1].

Human listeriosis is on the top five most commonly reported zoonosis under the surveillance of the European Union (EU) and presents the highest case fatality rate, that is, 16.2% [2]. The incidence of invasive forms of the disease is higher in risk groups, such as the elderly, immunocompromised individuals, pregnant women, and newborns. In countries with established surveillance programs, the incidence of listeriosis is reported to be increasing, and the distribution of cases is shifting, primarily affecting elderly persons. In 2016, most cases of listeriosis were reported in individuals over 64 years of age [2]. This is worrisome, as advances in the field of medicine are leading to growing life expectancies; therefore, an increased risk of foodborne listeriosis is expected to occur in the near future.

Listeriosis is an atypical disease with multiple routes of infection, including aerial, cutaneous, transplacental, nosocomial, direct contact, or digestive tract. However, surveillance studies and investigation of recent outbreaks have demonstrated that the most associated transmission pathway to humans is the intake of contaminated food (digestive tract). Ready-to-eat foods, particularly refrigerated foodstuffs, such as milk and dairy products, meat and meat products, raw vegetables, and fruits, have been related to recent outbreaks [3, 4].

The food industry relies on a variety of processing and preservation methods to produce safe and healthy products with adequate shelf life and that are appreciated by consumers. These methods inactivate or inhibit the growth of pathogenic microorganisms such as *L. monocytogenes* and suppress undesirable chemical and biochemical changes, thereby ensuring food safety and maintaining desirable physical and sensory properties. The methods currently used in food preservation involve physical, chemical, or biological factors. In combination with other strategies, refrigeration, freezing, addition of acidifying agents or curing agents (e.g., sodium chloride and sodium nitrite), radiation and high-pressure processing are the most reliable and used preservation techniques. However, there are studies which demonstrate that *L. monocytogenes* strains have mechanisms that allow them to survive and resist the stresses caused by these processing methods [5].

This review focuses on key issues such as the molecular mechanisms underlying *L. monocytogenes* survival and adaptation to stresses caused by different environmental conditions. Since many of the stresses can be found in both food and humans, we will try to correlate these molecular mechanisms with the organism's virulence. Studies on the development of technologies to control and prevent the contamination of *L. monocytogenes* in food matrices and food processing facilities are also briefly discussed.

2. Cold stress response

Cold stress adaptation is a fundamental characteristic of *L. monocytogenes* that markedly contributes to the microorganisms' dissemination via refrigerated food products. Although most foodborne pathogens are effectively controlled under cooling storage, *L. monocytogenes* proliferation persists so, cold-stored contaminated foods provide proper conditions for survival and growth of these organisms [6, 7].

L. monocytogenes, as a psychrotolerant bacterium, is able to grow over a wide range of temperatures (1–45°C), although the optimum temperature range is from 30 to 37°C [8]. Cold stress adaptation in *L. monocytogenes* is mediated through many molecular response mechanisms whose nature remains mainly vague, besides some aspects of this phenomenon have been clarified in model microorganisms.

2.1. Listerial mechanisms of low-temperature resistance

L. monocytogenes response to cold shock comprises the synthesis of cold-shock proteins (CSPs), while during balanced growth at low temperatures, it produces cold acclimation proteins (CAPs). Twelve CSPs and four CAPs were identified as a result of cold stress [9]. The main functions involving CSPs include chaperones involved in DNA recombination course, transcription, translation, and protein folding [10]. The cold adaptation of this pathogen is accompanied by gene expression changes. When cultured at 10°C, *L. monocytogenes* RNAs are increasingly synthesized compared to growth at 37°C [11]. A higher mRNA expression for chaperone proteases suggests that ClpP, ClpB, and GroEL enzymes may participate in the degradation of damaged or abnormal polypeptides arising due to growth at low temperatures.

Changes in temperature also lead to an alteration in the membrane lipid composition to maintain the ideal membrane fluidity required for proper enzyme activity and transport of solutes [12]. *Listeria* cell membrane contains high amounts of iso and anteiso, odd-numbered, branched-chain fatty acids (>95%). When grown under refrigeration temperatures, the anteiso-C15:0 represents 65–85% of total membrane fatty acids. When grown at 37°C, predominant fatty acids are anteiso-C15:0 (41–52%), anteiso-C17:0 (24–51%), and iso-C15:0 (2–18%) [13]. Growth at low temperatures also causes an increase of unsaturated fatty acids, which helps enhancing the fluidity of the membrane. Decreasing the growth temperature from 20 to 5°C precedes a switch from iso to anteiso branching (i-C15:0 to a-C15:0) and a fatty acid shortening (a decrease in C17:0). Annous et al. [13] suggested that the growth of *L. monocytogenes* in refrigerated foods could be controlled by food-grade agents inhibiting the biosynthesis of anteiso-C15:0.

L. monocytogenes growth at low temperatures is also stimulated by the presence of cryoprotectant compatible solutes, for example, betaine, glycine, and carnitine [14, 15]. *Listeria* imports and accumulates these solutes from the environment, and this is one of the functions of sigma factor σ^B (*Listeria*'s general stress transcription factor) during growth at low temperature [16]. In response to cold shock, σ^B controls the transcription of genes encoding the BetL, Gbu, and OpuC uptake system, involved in the accumulation of glycine, betaine, and carnitine. Studies with mutants having deleted osmolyte transporter genes demonstrated the cryoprotective activity of these compounds [17].

3. Acid stress response

L. monocytogenes may be exposed to high acidity levels while in the food chain and during gastrointestinal (GI) passage in the host (i.e., following exposure to fatty acids, in the phagosome of macrophages during systemic infection, and even upon exiting the host, due to fluctuations in environmental pH).

Being a neutrophile (optimum pH 6 or 7), *L. monocytogenes* keeps the intracytoplasmic pH close to neutrality, though pH oscillations in the external medium are imperative for its survival and a prerequisite for pathogenesis and infection [18]. Acid tolerance response (ATR) is the adaptive phenomenon that permits the pathogen to preserve pH homeostasis when exposed to low pH. Understanding the molecular mechanisms of acid adaptation and pH homeostasis is essential in order to control the pathogen growth in high-risk foods and predict the ability to cause disease.

3.1. Listerial mechanisms of acid resistance

Cellular exposure to pH stress induces the modulation of fatty acid profiles in *Listeria* cell membrane, although the changes differ from those documented for other genera [19]. In *L. monocytogenes*, larger proportions of linear chain fatty acids are incorporated into the membrane, with increased levels of C14:0 and C16:0 and a reported concomitant decrease in C18:0 [20, 21].

Under high acidic environments, two chaperonins (DnaK and GroES) and a serine protease (HtrA) have been identified and characterized in *Listeria*, being necessary for the organism survival [22–24]. Other studies shed light on the role of σ^B in modulating genes involved in pH homeostasis and gastrointestinal persistence, thus crucial in *L. monocytogenes* survival after exposure to acid conditions. It has been reported that *Listeria* mutants that lack a *sigB* functional gene exhibit a decreased resistance to low pH conditions, besides σ^B regulates the expression of OpuC, a cold-activated transporter for carnitine.

Additional mechanisms of acid resistance such as the F0-ATPase complex, arginine deiminase system (ADI), and the glutamate decarboxylase (GAD) have been elucidated.

3.1.1. F0F1-ATPase complex

F_0F_1-ATPase is an enzyme organized in two distinct although physically linked domains. The catalytic part (F_1) is cytoplasmic while the integral membrane domain (F_0) acts as a membrane channel for proton translocation. Cytoplasmic domain may either catalyze the synthesis of adenosine triphosphate (ATP) when the protons pass into the cytoplasm through the membrane-bound domain, or hydrolyze ATP when the protons move outside of the cell. Thus, the F_0F_1-ATPase complex is responsible for the aerobic synthesis of ATP, as a result of protons moving into the cell, and generates a proton motive force anaerobically by expelling protons. As a consequence of the latter mechanism, F_0F_1-ATPase is thought to increase intracellular pH in acidic situations [25].

3.1.2. Arginine deiminase system

This system comprises three enzymes: arginine deiminase (encoded by *arcA*) which catalyzes the hydrolysis of arginine to citrulline and ammonia; ornithine carbamoyltransferase (encoded by *arcB*) which is responsible for converting citrulline to ornithine and carbamoylphosphate, in the presence of phosphate; and carbamate kinase (encoded by *arcC*) which synthesizes ATP from carbamoylphosphate and adenosine diphosphate (ADP).

Arginine is transported into the cell in exchange for an ornithine molecule that is moved outside through the transporter encoded by *arcD*, while the pathway enzymes ultimately catabolize arginine to ornithine, ammonia, and CO_2. Ammonia is produced through the catabolization of arginine via the ADI system combined with intracellular protons to produce ammonium ions. This reaction increases intracellular pH, thus allowing survival in hostile environments that would otherwise be lethal to the cell [26]. In addition, ATP is generated by the system and this can be used for driving out protons through F_1F_0-ATPase [27].

3.1.3. Glutamate decarboxylase system

The GAD enzyme, generally encoded by *gadA* or *gadB*, irreversibly decarboxylates glutamate, producing the neutral γ-aminobutyrate (GABA). This reaction results in an increase of the cytoplasmic pH due to the consumption of an intracellular proton. GABA produced by the decarboxylation reaction is subsequently exchanged on the cell membrane for a glutamate molecule by a glutamate: GABA antiporter, generally encoded by the *gadC* gene [28].

The GAD system is crucial for *L. monocytogenes* acid adaptation and, consequently, for a successful passage through the gastric environment, a necessary condition for latter invasion of intestinal epithelial cells [29]. The loss of genes encoding a GAD enzyme and a glutamate transporter decreases the cell's ability to survive in low pH environments and consequently to cause infection [30]. Stress factors commonly associated with the GI tract (low pH, anaerobiosis, hypo- and hyperosmotic shock, bile salts, and chloride ions) have been shown to induce GAD system expression in a variety of bacteria [31, 32].

4. Osmotic stress response

Osmotic stress defines the osmotic strength variation of an organism environment, which results from desiccation or from a high content of osmotically active compounds (salt or sugars) in the environment, lowering its water activity (a_w). Since the bacterial cytoplasmic membrane is permeable to water but not to most other metabolites, hyper- or hypo-osmotic shock causes an efflux or influx of water, accompanied by a concomitant decrease or an increase in intracellular volume, respectively. In general, the internal osmotic pressure is higher than that of the surrounding medium, generating turgor, the driving force for cell extension, growth, and division. Therefore, the bacterial maintenance of pressure turgor is critical to survival in osmotic stress conditions.

The maximum NaCl concentration that permits *L. monocytogenes* growth ranges from 7 to 10% [33]. This osmotolerance is vital during its infectious cycle, since *L. monocytogenes* encounters elevated osmolarity in the food processing industry and in the gastrointestinal lumen of the host. The response of microorganisms to osmotic stress is called osmoadaptation and holds physiological changes and variations in gene expression patterns [34].

4.1. Listerial mechanisms of osmotic resistance

Compatible solute osmoadaptation is a biphasic response in which elevated levels of potassium cation K^+ (and glutamate, its counter-ion) represent a primary response, succeeded by a significant increase in cytoplasmic concentration of compatible solutes. Cells absorb osmolytes from the external environment to restore osmotic balance within cells. The solute-mediated osmoprotection stimulates the growth of cells subjected to high salt concentrations. Deletions of these osmolyte transporters reduce the growth of *Listeria* under conditions of hyperosmolarity [14, 30, 35]. In addition to previously mentioned compatible solutes (glycine, betaine, and carnitine), proline is important for the survival under hyperosmolarity conditions [36]. σ^B factor, as an important part of the overall stress response of *L. monocytogenes*, mediates the expression of *ctc* gene and the use of betaine and carnitine as osmoprotectors.

In response to osmotic stress, two genes involved in cell envelope modification have been identified: *lmo2085*, a putative peptidoglycan-linked protein, and *lmo1078*, a putative UDP-glucose phosphorylase that catalyzes the formation of UDP-glucose, a precursor of membrane glycolipids and of the cell wall [37].

A further mechanism of osmotic adaptation is the modification of genetic expression leading to an increased or a decreased synthesis of several proteins. Salt-shock proteins are rapidly induced and overexpressed for a short time period, being similar to those induced in cold-shock response (CSPs and CAPs). Among CSPs induced in *L. monocytogenes*, there are two general stress response proteins, DnaK that acts as a heat-shock protein stabilizing cellular proteins and Ctc that is involved in high osmolarity resistance in the lack of osmoprotectants, such as glycine, betaine, and carnitine, in the medium [38]. Additional stress response proteins, including ClpC (an ATPase), ClpP (a protease), and HtrA (a protease), are essential for osmotic and acid stress adaptation in *L. monocytogenes* [39]. HtrA may play a role in degrading misfolded proteins and is beneath LisRK control, a two-component regulatory system important for osmoregulation [36].

5. Desiccation stress response

Desiccation tolerance defines the bacteria's aptitude to survive for extended periods on a surface, deficient of nutrients and water. As so, *L. monocytogenes* desiccation tolerance is most likely associated with the ability to persist in food production surfaces and consequently cross-contaminate food products [40]. The low a_w resulting from high osmolarity decreases turgor pressure in a bacterial cell inhibiting bacterial growth [41]. Drying and addition of salt or sugar are traditional methods to lower food a_w and therefore enhance its prolonged shelf life. *L. monocytogenes* grows optimally at $a_w \geq 0.97$, although it may survive in foods with low a_w [42]. When compared to other common infectious foodborne pathogens, *L. monocytogenes* does not appear to grow at $a_w < 0.90$ but it can survive in these conditions, particularly under refrigeration, for long periods. To date, existing information regarding *L. monocytogenes* desiccation survival is limited and primarily focuses on factors influencing the survival to osmotic stress [40, 43–46]. Strains of serotypes 1/2c and 1/2b were the most tolerant to desiccation, followed by 4b and 1/2a [47]. Hansen and Vogel [46] showed the protective effect of osmoadaptation and also the formation of biofilms on the desiccation survival.

6. High hydrostatic pressure

A high hydrostatic pressure (HHP) represents the application of pressure in the range of 50–1000 MPa, though the inactivation of vegetative cells of bacterial species is typically reached from 300 to 700 MPa, and bacterial spores inactivation demands higher pressure levels up to 1000 MPa [48]. However, depending on the pressure level, HHP treatments can fully inactivate bacteria or impose sublethal injuries. For pressures up to 400 MPa, the integrity of Gram-positive bacterial cells and metabolic activity are maintained, with very limited cell destruction [49]. Over the last years, it has been stated that *L. monocytogenes* is potentially capable of recovering culturability following HHP exposure [49–52]. Physiological studies have also demonstrated that increasing pressure levels results in an accelerated decline of metabolic indicators, such as the activity of the LmrP membrane transport system [53]. These findings suggest that bacteria exposed to HHP are unable to grow due to cell injury, but yet can mount a nonspecific response to high pressure. A proportion of the cell population is able to maintain cellular activity of some kind after HHP, demonstrating the capacity to cellular repair and regrow, when adequate conditions are available [49].

To date, little research has been conducted regarding the mechanisms of bacterial adaptation and resistance to high pressure. Wemekamp-Kamphuis et al. [54] demonstrated that one of the responses that enable *Listeria* survival upon HHP treatment results from induction of the general stress response mediated by σ^B. *L. monocytogenes sigB* deletion mutant was more susceptible to HHP exposure than the wild type, while induction of σ^B resulted in an increased HHP protection relative to the untreated control strain.

Several pressure-induced proteins have been increasingly synthesized when compared to the synthesis of other control proteins at atmospheric pressure [55]. *L. monocytogenes* has shown to actively express many genes as a response to high pressure, but some functional categories appear more affected than others. Genes that tend to be expressed at higher levels under high pressure are genes encoding for transport and binding, signal transduction and chemotaxis, cellular processes, transcriptional regulators, metabolism, and protein fate [56]. The stabilization and maintenance of the bacteria cell is at high focus, showed by the significant regulation of ribosomes and proteins, together with components involved in the cell envelope and the septal ring. It is assumed that the activation of genes involved in the lipid and peptidoglycan biosynthetic pathways is connected to this function. Upregulation of genes associated with generalized repair and maintenance has been proved, where the activation of cold- and heat-shock genes is an example for this [57, 58]. When high pressure demands more energy to be used on repair, energy production and conversion is suppressed. The repression of several energy production/conversion, carbohydrate, and other carbon compound catabolic genes may represent a diminishment of catabolism in cells imposed by HPP treatments. This can be seen by the pressure-induced switch from active growth to a cell repair state, the stationary phase, resulting in a decreased growth rate [59].

Several genes associated with cell formation and shape, as well as synthesis or reassembly of cell-wall constituents, in particular peptidoglycan and fatty acids, were observed to have an increased expression. Because of this, genes involved in such functions can be considered as very central in the response to high pressure. It is presumed that *L. monocytogenes* increases both cell division and cell-envelope-associated gene expression aiming to replace damaged components and thus compensate membrane and wall damages [59].

Cell membranes damage by HPP may possibly be a main cause of inactivation or death in Gram-negative bacteria, but it is fallacious to admit that in Gram-positive bacteria. Cell membrane and wall stabilization in the stationary growth phase do provide a protective effect against HPP, being a major factor for the survival of HPP-induced damage [60]. Beyond cell envelope damage, HPP interferes within the nascent septal ring formation along with other associated cell-wall formation and chromosome segregation processes [59].

7. Stress impact on *L. monocytogenes* virulence

L. monocytogenes has a profound ability to adapt to unfavorable stressful environments, switching from a saprophyte to an intracellular pathogen capable of causing serious infection to the host [61]. In this transformation, σ^B dominates both in the external environment and during gastrointestinal transit, while positive regulatory factor A (PrfA) plays a central role on the intracellular infection. In concert with PrfA, σ^B activates the transcription of several *L. monocytogenes* virulence genes: (1) *bsh*, encoding bile salt hydrolase, essential in gastrointestinal colonization prior to invasion; (2) *inlA*, encoding internalin A, mediates entry into human intestinal epithelial cells; and (3) *gadA*, encoding part of the glutamate decarboxylase system, crucial for acid survival [62]. σ^B also contributes to the transcriptional activation of *prfA*, encoding PrfA, a central virulence regulator of virulence gene expression in *L. monocytogenes* [63].

PrfA-dependent virulence gene cluster or LIPI-1 (*Listeria* pathogenicity island 1) encodes most virulence factors involved in the pathogenic infectious cycle. This chromosomal locus comprises the following genes: (1) *hly*, encoding listeriolysin O (LLO), a pore-forming toxin crucial in the escape from phagocytic vacuoles; (2) *plcA* and *plcB*, encoding two phospholipases C which cooperate with LLO in the escape from bacterial phagosomes; (3) *mpl*, encoding a metalloprotease implicated in the maturation of proenzyme pro-PlcB; (4) *actA*, encoding ActA protein involved in the intra- and intercellular motility of the bacteria; and (5) *prfA*, encoding PrfA, a transcriptional activator of LIPI-1 genes [64]. The expression of additional genes dispersed on the chromosome may be PrfA-regulated, as the internalin locus *inlAB* [65], the genes encoding internalins InlA and InlB cell-wall-anchored proteins which induce *Listeria* phagocytosis [66].

Following the complete genome sequencing of several *L. monocytogenes* strains, an increasing number of virulence-related proteins are being identified and their specific involvement during infectious stages deciphered (**Table 1**).

In addition to other factors, the infectious potential of *L. monocytogenes* is conditioned by the environmental conditions prior to host invasion. A correlation between stress response and virulence seems to exist and associates strains having more effective stress response mechanisms to being also more virulent [84]. Early studies by Durst [84] and Wood and Woodbine [85] demonstrated that cold storage may enhance virulence of some strains because the

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Involvement	Proteins/function	Ref.
Regulation	**PrfA** *Positive regulatory factor A, central virulence regulator of virulence gene transcription.*	[68]
	SigmaB (σ^B) *General stress transcription factor.*	[69]
	CtsR *Class III stress-response regulator, a transcription repressor.*	[70]
	HrcA *Heat regulation at controlling inverted repeat of chaperone expression elements. A transcription repressor.*	[71]
Attachment and invasion	**InlA** *Internalin A, surface protein that mediates entry into cells expressing its receptor, the E-cadherin.*	[65]
	InlB *Internalin B, surface protein that mediates entry into cells expressing one of the receptors gC1qR, HGF-SF, Met, and the glycosaminoglycanes (GAGs).*	[72]
Lysis of vacuoles	**LLO** *Listeriolysin O, hemolysin required for vacuole escape by lysis of the phagosome membrane.*	[73]
	PC-PLC *Phospholipase activated by proteolytic cleavage involving Mpl or by cellular proteases. Required for the lysis of the double-membrane vacuole.*	[74]
	Mpl *Metalloprotease required for the maturation of PC-PLC.*	[75]
Intracellular multiplication	**Hpt** *Hexose phosphate transporter required for intracytosolic proliferation.*	[76]
Cell-to-cell spread	**ActA** *Actin assembly-inducing protein, involved in cell-to-cell spread.*	[77]
Environmental stress response and virulence	**HtrA** *Serine protease involved in acid and osmotic stress response.*	[78]
	Bsh *Bile salt hydrolase involved in the intestinal and hepatic phases of listeriosis.*	[79]
	ClpC *ATPase protein promoting early bacterial escape from the phagosome of macrophages and thus virulence.*	[80]
	ClpP *Serine protease involved in proteolysis and required for growth under stress condition.*	[81]

Involvement	Proteins/function	Ref.
	DnaKJ	[22]
	Chaperone heat-shock proteins encoded by the dnaK operon and required for phagocytosis.	
	GroES, GroEL	[23]
	Chaperone proteins which regulate HrcA posttranscriptionally.	
	GAD	[29]
	The glutamate decarboxylase system, involved in acid stress response.	
	BetL	[82]
	Glycine betaine transport system I, involved in osmotic stress response.	
	Gbu	[15]
	Glycine betaine transport system II, involved in osmotic stress response.	
	OpuC	[83]
	Carnitine transport system, involved in cold and osmotic stress response.	

Table 1. Stress response and virulence-associated proteins in ***Listeria monocytogenes*** (adapted from reference [67]).

pathogen virulence rather increases when grown under refrigeration than at optimal growth temperature. By contrast, virulence gene expression was reported to be downregulated at temperatures below 30°C, besides PrfA is only formed at 37°C [85]. According to Loh et al. [86], the expression of *prfA* is nearly 16-times higher at 37°C compared to that at 30°C, and imperceptible in cells cultivated at 20°C. The specific pathogenicity of LLO can be fully recovered in less than 24 h by incubating refrigerated cells at 37°C [87]. This virulence recovery after heat shock reinforces the importance of eliminating *L. monocytogenes* from minimally processed ready-to-eat foods held at refrigeration temperatures for long periods.

Low pH and high salt content are common factors often found in foods contaminated with *L. monocytogenes* [89]. Even though at these conditions, the growth of most foodborne and spoilage bacteria is restricted, *L. monocytogenes* is capable of surviving and even grow in such environments; long-term adaptation to these sublethal stress conditions results in altered virulence [88].

Conte et al. [31, 89] demonstrated that short-term exposure (1 h) of *L. monocytogenes* to a sublethal acidic environment (pH 5.1) not only increased its invasiveness to the human colon adenocarcinoma cell line Caco-2 but also increased the ability of *L. monocytogenes* to survive and proliferate in macrophage-like cells, suggesting that exposure to a low pH (e.g., in the human stomach) may enhance listerial overall virulence. In addition, LLO excreted by virulent *L. monocytogenes* showed a maximal activity at pH 4.0–5.0. In another study, the exposure of *L. monocytogenes* to acidic shock has induced the transcription of two important virulence genes (*inlA* and *bsh*) [90]. Conversely, a study by Rieu et al. [91] reported a decrease in virulence gene transcription after 5 h at pH 4.0 achieved with acetic acid. This conflicting finding may be sustained by the use of organic acids since they might be more harmful to the bacteria. Some weak organic acids enhance pathogenicity of the bacterium, while others reduce it, as the secretion of LLO is increased by citrate, acetate, and lactate, whereas sorbate inhibited this hemolysin [92]. This knowledge would be important for the selection of acidulants to be used in different foods.

Garner et al. [93] reported an intensified invasiveness of *L. monocytogenes* for Caco-2 cells when grown at 7°C rather than at 37°C, and, for both temperatures, the invasion ability was greater in cells grown at pH 7.4 compared to growth at pH 5.5. A growth temperature of 37°C, pH 7.4, in the presence of NaCl or sodium lactate, enhanced *L. monocytogenes* invasiveness; however, the pre-exposure to gastric fluid (pH 4.5), even for as short as 10 s, substantially reduced its invasion. These findings intimate that listerial virulence-associated characteristics seem to be affected by specific food properties (e.g., the presence of organic acids or salt). The authors further showed that *L. monocytogenes* growth phase affects its ability to invade Caco-2 cells. The invasion by log-phase cells was 9.5-fold lower than invasion by stationary-phase cells, corroborating other studies which demonstrate that exposure of *L. monocytogenes* to different environmental conditions can change invasiveness and virulence [93]. Accordingly, the increased stationary-phase invasiveness also coincides with stationary-phase induction of σ^B activity [90]. In stationary-phase cells, *inlA* expression is regulated in a σ^B-dependent manner, and growth phase-dependent effects on invasion appear independent of PrfA [94, 95], contributing to *inlA* transcription [96].

Complementary studies demonstrate that *L. monocytogenes* pathogenicity requires an adaptive acid tolerance response, so the ability to survive gastric acid fluid and to invade host cells is related to ATR activation [30, 89, 97]. This finding is supported by the fact that the glutamate decarboxylase (GAD) system, as the ATR most important component, is required for listerial survival in the gastric environment, and also LisRK deletion, a two-component system involved in acid resistance regulation, caused a dramatic reduction in virulence [29, 98].

A further prerequisite for *L. monocytogenes* infection depends on the ability to counteract conditions of elevated osmolarity in the gastrointestinal tract. As mentioned in Section 2.1, the carnitine uptake system (OpuC) is directly linked to osmotic stress resistance of *L. monocytogenes* and to its ability to reach and proliferate in the liver and spleen [17]. Carnitine (produced from the desquamation of the gastrointestinal epithelial layer) was formerly proved to act as a crucial osmoprotectant, facilitating growth in this gastrointestinal environment, once changing the carnitine transported OpuC resulted in a significant reduction in *Listeria* ability to colonize the upper small intestine and cause subsequent systemic infection [99, 100]. A supporting study by Wemekamp-Kamphuis et al. [17] demonstrated that a triple mutant, defective in all three compatible uptake systems (BetL, Gbu, and OpuC), showed a similar phenotype to that of a single opuC mutant, mutually revealing a decreased ability to cause systemic infection relative to the parent. Those were clear evidences that *betL* and *gbu* do not play a significant role in *L. monocytogenes* pathogenesis and that it is the carnitine uptake system that most induces listerial virulence. In addition, Joseph et al. [101] also identified OpuCA and OpuCB as being induced intracellularly. Since the contribution of each transporter is dependent on the external environment, there are occurrences when each system is tailored for optimal effects within a certain environmental niche.

Over the last years, novel trends in food production tend to preserve the natural flavor and texture of products using minimal processing. Non-thermal food preservation usually allows a significant microbial reduction, and mounting evidence also demonstrates that the conditions applied by alternative technologies may influence bacterial virulence [102]. The application of HHP has been shown not to induce mutations in the internal genes, *inlA* and *inlB*, implicated in the adhesion and internalization of *L. monocytogenes* in human cells. However, when the effect of HPP on the *ctsR* gene is observed, a reduction in virulence potential of surviving cells was noted. Likewise, virulence and reduced motility may be the result of a mutation in this gene corresponding to the loss of a single amino acid. This suppression could be related to a high-pressure tolerance [70, 103].

8. Conclusions

Exposure of *L. monocytogenes* to sublethal environmental stresses can enhance its survival to subsequent lethal conditions and additionally induce the expression of the organism's virulence genes. Therefore, exposure of *L. monocytogenes* to food-associated stresses such as high salt concentrations or low temperatures during refrigerated storage may result in increased virulence and thus a higher risk for listeriosis. Any strain of *L. monocytogenes* present in food is actually considered equally pathogenic. However, results from several studies support the idea that the heterogeneity among strains regarding the response to stress and virulence potential should be considered, once responses to food matrix and storage conditions are often strain specific.

Although significant advances in our understanding on stress response and virulence potential have been achieved in the last years, there is still a need to fulfill knowledge gaps on molecular mechanisms behind *L. monocytogenes* response to stress and virulence. Further studies on the influence of food matrix on stress tolerance and virulence potential of different strains, recovered from foods and from patients, are needed. This information can be further used by regulators to refine previous risk assessments and also in the definition of control measures by the food industry.

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